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Hydrogen Exchange Rates in Pancreatic Trypsin Inhibitor Are Not Correlated to Thermal Stability in Urea†

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ABSTRACT: The hydrogen–isotope exchange rates of single, assigned peptide amide NH protons have been reported for bovine pancreatic trypsin inhibitor (BPTI). We have interpreted the complex pH and temperature dependence of the single proton exchange rates of BPTI as arising from changes in the mechanism between two processes that differ in temperature dependence [Hilton, B. D., & Woodward, C. K. (1979) *Biochemistry 18*, 5834; Woodward, C. K., & Hilton, B. D. (1980) *Biophys. J. 32*, 561]. One process, characterized by an activation energy of 20–35 kcal/mol, involves motions of the folded state that allow exchange of interior protons. The second process, characterized by an activation energy of ~65 kcal/mol, corresponds to major, cooperative unfolding. This two-process model explains all of the unusual features of the pH and temperature dependence of the kinetics of the slowest exchanging protons and differs fundamentally from the interpretation of the same data by Wuthrich & Wagner (1979) [Wuthrich, K., & Wagner, G. (1979) *J. Mol. Biol. 130*, 1]. For example, in the two-process model, characteristics of the high activation energy exchange rates cannot be ascribed to internal motions that limit exchange from the folded state. The model predicts that high activation energy exchange rates correlate with thermal stability of the protein, while low activation energy exchange rates may not. As a further test of the model, we have measured the pH and urea dependence of the major BPTI thermal unfolding transition and the effect of 8 M urea on low activation energy exchange rates. In support of the two-process interpretation of the BPTI single proton exchange data, we find the following: (1) The enthalpy of unfolding in 8 M urea, 45 ± 5 kcal/mol, agrees well with the value of ~45 kcal/mol obtained from the proton exchange analysis. (2) BPTI low activation energy exchange rates are not correlated to thermal stability in 8 M urea. (3) Between pH 4 and 2.5, BPTI titrates between two isomers of the folded state that differ in thermal stability. This transition, and the two-process model, explains the unusual pH dependence of the single proton exchange data, particularly the shallowness of the pH–rate curve and the abnormally high pH of the minimum rate, pH_{min}.

For folded proteins, the hydrogen–isotope exchange kinetics of peptide amide NH protons with solvent measure internal motions of the tightly packed conformation. Exchange rates for NH's within a single protein may vary over a range of >8 orders of magnitude, with the rates at the fast end of the distribution on the order of those of randomly coiled polypeptides. Exchange kinetics of folded globular proteins demonstrate that although interior NH atoms are shielded from solvent, there are conformational motions by which most buried backbone NH protons are rendered accessible to solvent OH^- and H^+ ions and H_2O. Understanding the nature of these motions is the object of hydrogen exchange studies (Woodward & Hilton, 1979, 1980; Englander & Englander, 1978; Englander et al., 1980).

Hydrogen–isotope exchange in proteins has most often been reported as total exchange, the average exchange kinetics of all labile protons measured simultaneously (Englander & Englander, 1978; Hvidt & Pedersen, 1974; Nakanishi & Tsuboi, 1974; Englander et al., 1979). With these kinds of data, there is the drawback that one cannot derive individual rate constants nor assign specific rates to individual NH groups. Advances in the analysis of total NH exchange kinetics have been made in the application of a distribution function (Knox & Rosenberg, 1980), and in the assignment of exchange rates to known peptide sequences (Rosa & Richards, 1979, 1981). However, the most specific approach to the quantitative analysis of hydrogen exchange kinetics is to measure the exchange rates of single protons by NMR spectroscopy. Extensive single proton exchange data have been reported for one set of protons, the eight slowest exchanging NH protons in bovine pancreatic trypsin inhibitor (BPTI) (Hilton & Woodward, 1978, 1979; Wuthrich & Wagner, 1979; Richarz et al., 1979), and in BPTI homologues and derivatives (Wagner & Wuthrich, 1979a). Assignments of these NH's have been made (Dubs et al., 1979). Our interpretation of these single proton exchange data differs fundamentally from the interpretation of Wuthrich and co-workers.

We have proposed that the observed exchange rate of each NH proton is the sum of its rates for exchange by two processes that differ widely in temperature dependence. One

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process involves major thermal unfolding and is characterized by a high activation energy (E^*_{app}), which in BPTI is ~65 kcal/mol. For the slowest exchanging BPTI protons, this mechanism dominates at lower pHs and higher temperatures. The observed exchange rate is equal to the unfolding rate in the pH-independent region of the pH–rate curve for the two slowest exchanging protons (Woodward & Hilton, 1980).

The second process involves motions of the folded macrostate and is characterized by activation energies of 20–35 kcal/mol. For the eight slowest exchanging protons in BPTI, this is the predominate mechanism at higher pHs and lower temperatures. For the more rapidly exchanging BPTI NH protons, this is the main mechanism at lower temperatures and at all pHs over the pH range 2–10.

For the mechanism of the low E^*_{app} process, we propose small fluctuations of interior atoms on the order of tenths to ~1 Å, which provide pathways for the movement of solvent into the protein interior where isotope exchange takes place (Woodward & Rosenberg, 1971b; Woodward, 1977; Woodward & Hilton, 1980). The apparent enthalpies for the protein internal fluctuations in this mechanism range from 0 to 15 kcal/mol, assuming 20 kcal/mol for the activation energy chemical exchange step.

Regardless of the mechanism of the low E^*_{app} process, the two-process model means that to characterize motions of the folded macrostate from hydrogen exchange data one must refer to exchange rates that have no contribution from the thermal unfolding process, i.e., to exchange rates with E^*_{app} of 20–35 kcal/mol.

Wuthrich and co-workers interpret the exchange data for the slowest exchanging protons in BPTI differently. For a series of BPTI derivatives and homologues, they observe an inverse correlation between thermal unfolding temperature and exchange rates measured under conditions where $E^*_{app} \simeq 65$ kcal/mol. Taking this together with the lack of a correlation between thermal lability and the rates of aromatic ring flips, they propose that the types of motions that accommodate ring flips are different from the motions allowing NH isotope exchange. This idea is incorporated into a model for motions of hydrophobic clusters (Wagner & Wuthrich, 1979a). Their basic assumption is that exchange rates, in general, are correlated with thermal stability. However, a correlation of thermal lability with low E^*_{app} exchange rates has not been shown. The correlation referred to by Wuthrich and coworkers is for high E^*_{app} exchange rates and indicates that major unfolding is rate limiting for the high activation energy process. As such, the correlation does not relate to the mechanism of exchange from the folded state, i.e., the low activation energy exchange process.

In this paper, we present data for three additional points that support the two-process interpretation of BPTI single proton NH exchange rates. (1) The enthalpy of the BPTI thermal unfolding transition obtained from the two-process model compares well with the value obtained from circular dichroism measurements of thermal unfolding in 8 M urea. (2) We observe that in the acid pH range BPTI undergoes a transition between two folded forms that differ in thermal stability. This transition explains the unusual shape of the pH–rate curve of the slowest exchanging BPTI NH protons measured by NMR. (3) We observe no correlation in BPTI between low activation energy exchange rates and thermal stability in 8 M urea.

Materials and Methods

BPTI was purchased from NOVO Industries (Denmark) and purified of low molecular weight contaminants by dialysis against water. The hydrogen–tritium exchange experiments in Figure 1 were carried out as described in Woodward et al. (1975a). Protein at 20 mg/mL was in-exchanged at pH 12.5, 25 °C, for 100 min in 0.3 M KCl, using KOH to adjust the pH (Snyder et al., 1975). The tritiated protein was then refolded by lowering the pH to 6.5 with HCl. The zero-time filtration was in Sephadex G-25 equilibrated with 0.1 M phosphate and 0.3 M KCl, pH 6.5, at 4 °C. The eluted protein-containing fractions were pooled and incubated in a water bath at the temperature indicated, and aliquots were removed for the determination of H/molecule remaining unchanged, H_{rem}, as a function of time. For the calculation of H_{rem}, protein concentrations were determined from $E^{1\%,1cm}_{280nm} = 7.9$ (Laskowski & Laskowski, 1954).

For the tritium–hydrogen exchange experiments testing the effect of 8 M urea (Figure 7), protein concentrations were determined as described in Barksdale & Rosenberg (1978) by trace labeling the protein with [^{14}C]formaldehyde (Rice & Means, 1971). Otherwise, the in-exchange procedure and the filtration procedures were the same as above.

Circular dichroism (CD) was measured with a Jasco J41C spectropolarimeter, with a jacketed cell of 5-mm path length, regulated to ±0.2 °C and at a protein concentration of 0.2 mg/mL. For each point, separate protein samples were used. Reversibility was checked by measuring the ellipticity at ambient temperature. The temperature inside the cell was monitored with a digital readout thermister and checked before and after each point. For the experiments in Figures 5 and 6, the samples for each data point were made from dilutions of a common stock. The mean residue weight ellipticity, $[\theta]$, at 225 nm was determined from the relationship

$$[\theta]_{225} = \frac{\theta_{obsd} MRW}{10dC}$$

where θ_{obsd} is observed ellipticity in degrees, MRW is the mean residue weight of 111 for BPTI, d is the path length in centimeters, and C is the protein concentration in grams per milliliter (Adler et al., 1973). The low values of $[\theta]_{225}$ reflect the low percentages of secondary structure determined from the X-ray crystal structure, 26% α helix and 33% β sheet (Chou & Fasman, 1974).

Results

The well-known thermal stability of BPTI is illustrated in Figure 1. At 90 °C in KCl, pH 4–8, the protein is only about one-third of the way through the transition. In 8 M urea, the protein is destabilized, and the complete thermal unfolding transition can be measured (Figure 2). Between pH 4 and 8, the midpoint of the transition, T_m, is 81 °C in 8 M urea as contrasted to T_m >95 °C in KCl. The enthalpy of the unfolding transition in 8 M urea is 45 ± 5 kcal/mol.

In both KCl and 8 M urea, the thermal stability is pH dependent. There are apparently three forms of folded BPTI that differ in thermal stability, one at pH <3, a second at pH 4–8, and a third at pH >10. The pH 4–8 form is the most stable. The transition to the less stable form at low pH is illustrated in the variation of the ellipticity at 225 nm vs. pH at 88 °C in KCl (Figure 3) as well as in the curves of Figure 1.

In 8 M urea, T_m also is constant over the pH range 4–8 and decreases between pH 4 and 2.5. However, at pH <2.5, the protein becomes more stable and the T_m rises (Figures 2 and 4). Figure 4 shows the ellipticity at 225 nm as a function of pH at 70 °C.

The total hydrogen–tritium exchange kinetics (Figure 5) are the average kinetics for the entire distribution of BPTI

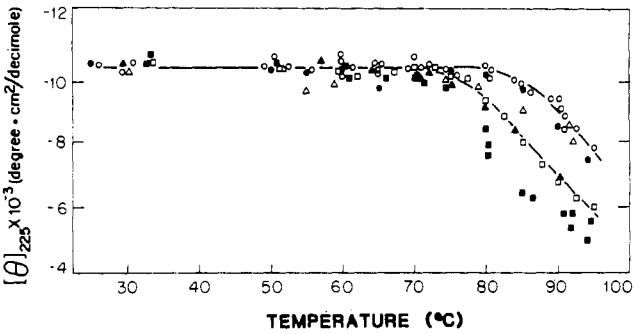

FIGURE 1: The thermal unfolding of BPTI in 0.3 M KCl measured by the mean residue weight ellipticity at 225 nm, $[\theta]_{225}$, as a function of temperature at pH 1 (■), 2.5 (□), 3 (▲), 4 (●), 8 (○), and 10 (△).

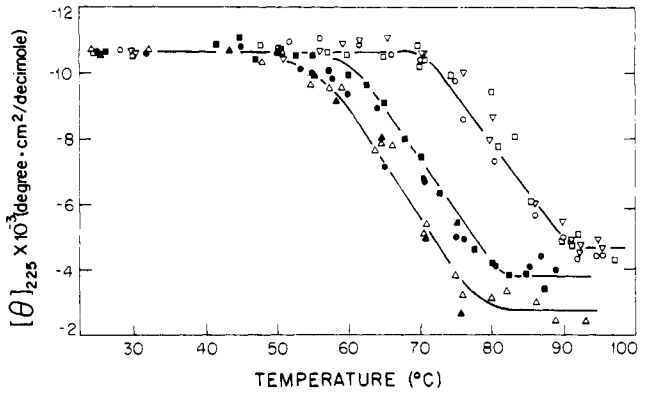

FIGURE 2: Thermal unfolding transition of BPTI in 8 M urea measured by the MRW ellipticity at 225 nm, $[\theta]_{225}$, as a function of temperature at pH 1 (●), 1.5 (■), 2.5 (▲), 3 (△), 4 (□), 8 (○), and 10 (▽).

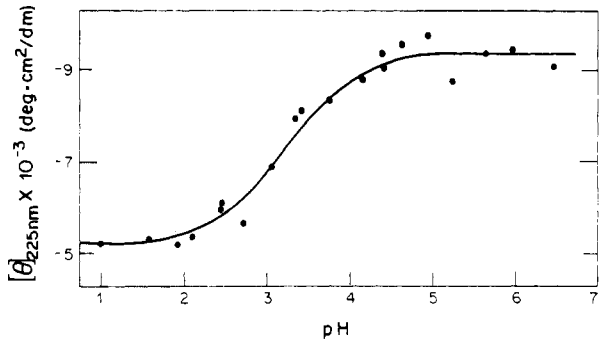

FIGURE 3: pH dependence of BPTI thermal stability in 0.3 M KCl, 88 °C. The MRW ellipticity at 225 nm, $[\theta]_{225}$, is proportional to the fraction of protein folded. The curve is calculated for the titration relationship $[\theta]_{225,pH} = ([\theta]_{225,HA} + [\theta]_{225,A^-}10^{pH-pK_a})/(1 + 10^{pH-pK_a})$ computed for $pK_a = 3.2$.

exchange rates. The apparent change in the number of H/molecule remaining unchanged, H_{rem}, actually observed in the experimental time window at a single temperature is a small fraction of the total labeled NH's. As the temperature is raised, an increasing number of protons become too fast to measure; the same values of H_{rem} are observed only over a relatively narrow temperature range. Between temperature intervals for which the same values of H_{rem} are measured, one can estimate E^*_{app} from pairs of curves in Figure 5 (Woodward & Rosenberg, 1971a; Wickett et al., 1974). These are shown in Figure 6. The E^*_{app} values gradually increase with temperature. Similar behavior is observed from RNase (Woodward & Rosenberg, 1971a), trypsin (Woodward et al., 1975a), and soybean trypsin inhibitor (Ellis et al., 1975) and is attributed to an increasing contribution to overall exchange from the thermal unfolding process. RNase S protein fragment shows a similar increase in E^*_{app} with temperature, but the

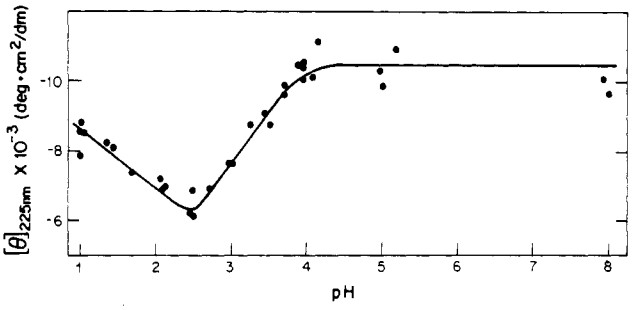

FIGURE 4: pH dependence of BPTI thermal stability in 8 M urea, 70 °C. The MRW ellipticity at 225 nm, $[\theta]_{225}$, is proportional to the fraction of protein folded.

values of E^*_{app} >35 kcal/mol are observed at lower temperatures, ~15 °C (Rosa & Richards, 1981). We suggest that the reason for the increase in E^*_{app} with temperature for the S-protein fragment is the same as for other globular proteins and that the low temperatures for the change in mechanism reflect the increased thermal lability of the S fragment as compared to that of typical globular proteins.

For BPTI total exchange, at temperatures <45 °C, E^*_{app} varies between 15 and 27 kcal/mol (Figure 6). Likewise, for the BPTI single proton data, E^*_{app} for exchange from the folded state ranges from 17 to 38 kcal/mol, with all but one in the range 17–30 kcal/mol [Table I in Woodward & Hilton (1980)].

For total hydrogen–tritium exchange, at temperatures >45 °C, E^*_{app} rises with temperature as the proportion of NH's exchanging by the unfolding process increases (Figure 6). For the single proton hydrogen–deuterium data, exchange by the thermal unfolding process has an $E^*_{app} \simeq 65$ kcal/mol (Woodward & Hilton, 1980). The high-temperature values of E^*_{app} for total tritium exchange do not reach 65 kcal/mol because the H_{rem} value is an average of a large number of fractionally labeled NH's. This is evident in a comparison of the composite kinetics of exchange for the slowest exchanging single protons at 68 °C, pH 6.5, constructed by summing the individual rates measured by NMR (dashed line, Figure 5 and inset). Comparing the tritium exchange curve at 65 °C with the deuterium exchange curve at 68 °C, one sees that for total exchange with tritium, pronounced curvature remains even for log plots and at very low values of H_{rem} because a portion of a very large distribution of rates is being measured.[1]

The addition of 8 M urea results in a small decrease in low E^*_{app} exchange rates of BPTI, pH 6.5, at temperatures from 0 to 40 °C. The data for 20 and 35 °C are shown in Figure 7. The activation energy for the interval 20–35 °C in 8 M urea is 25 kcal/mol, demonstrating that under these conditions we have not induced contributions from unfolding by the addition of urea.

The small decrease in the low E^*_{app} exchange rates in 8 M urea is similar to that observed for RNase with urea and other denaturants (Woodward et al., 1975a). This is not an effect of the denaturant on the protein conformation, since oxidized

[1] Comparison of the composite kinetics of the seven individually measured hydrogen–deuterium exchange rates, and the hydrogen–tritium exchange kinetics for H_{rem} values less than 7 at the same temperature (inset of Figure 5), demonstrates an additional point. This is that one cannot simply count, from the kinetics of total exchange, the average number of H_{rem} with apparent rates slower than model compounds and equate this number with a discrete set of NH's, e.g., NH's in intramolecular hydrogen bonds (Englander et al., 1972, 1979). A further point against that assumption is that there are cases of slowed NH exchange rates for peptide protons known not to be involved in H bonding (Llinas et al., 1973; Brewster & Bovey, 1971).

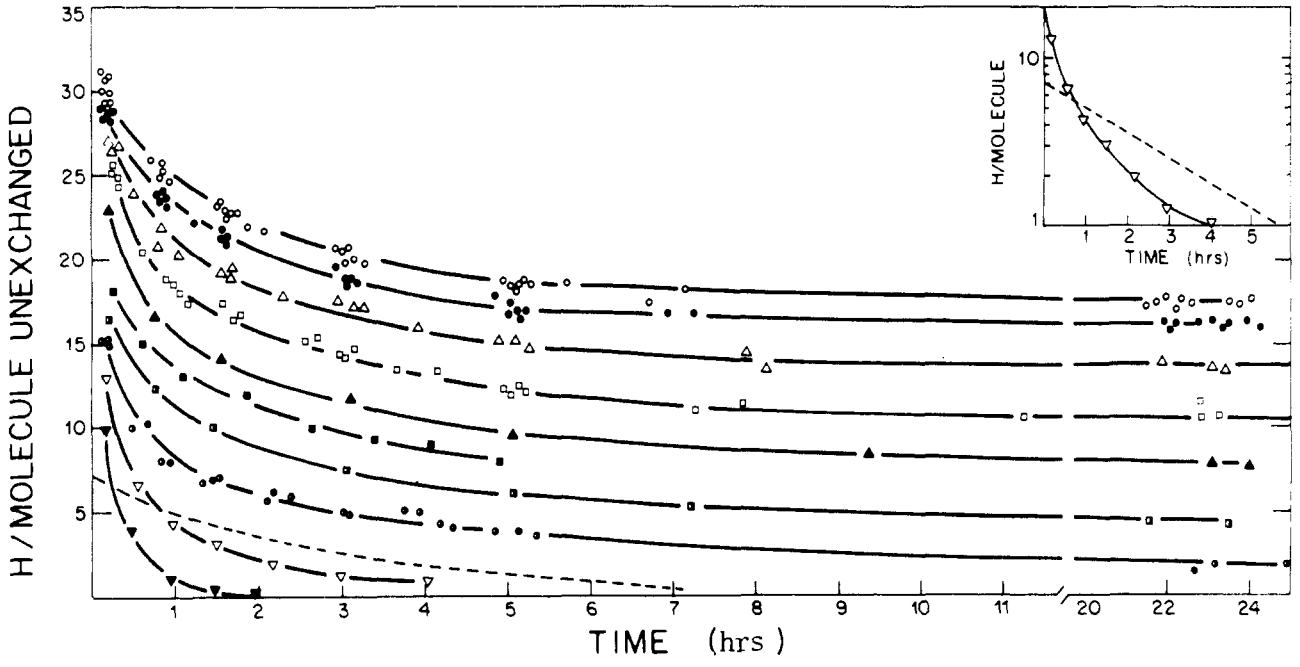

FIGURE 5: Hydrogen–tritium exchange kinetics of BPTI in 0.3 M KCl and 0.1 M phosphate, pH 6.5, at 20 (○), 25 (●), 30 (△), 35 (□), 40 (▲), 45 (■), 50 (◨), 55 (◑), 60 (▽), and 65 °C (▼). The dashed line represents the sum of the individual rates for the seven slowest exchanging protons at pH 6.5, 68 °C, obtained singly for hydrogen–deuterium exchange measured by NMR (Hilton & Woodward, 1979). No adjustment for the isotope effect has been made.

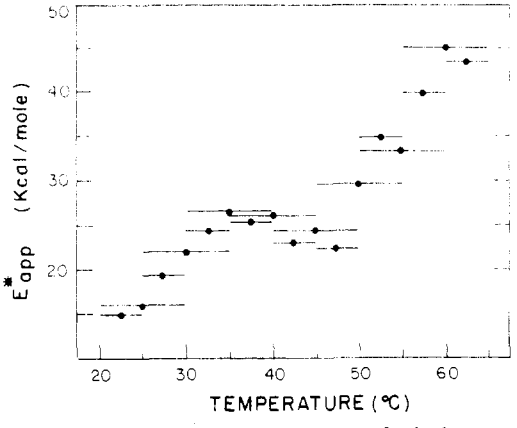

FIGURE 6: Apparent activation energies, E^*_{app}, for hydrogen–tritium exchange of BPTI. The values of E^*_{app} are calculated from pairs of curves in Figure 1, as explained in the text. The bar shows the temperature interval over which each activation energy was calculated; the circle is the average E^*_{app} for four to eight values of H/molecule remaining unexchanged.

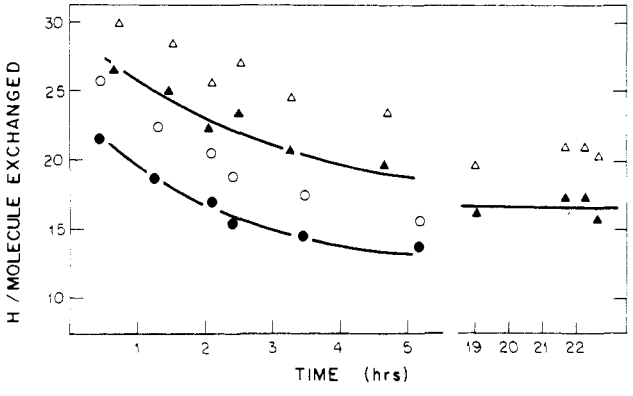

FIGURE 7: Total tritium–hydrogen exchange kinetics of BPTI with (open symbols) and without (closed symbols) 8 M urea in 0.3 M KCl and 0.1 M phosphate, pH 6.5, at 20 (△) and 35 °C (○).

RNase, an irreversibly unfolded protein, also shows decreased exchange rates in these solvents (Woodward et al., 1975a).

The effect of urea on exchange at higher temperatures cannot be measured with these types of experiments since at ~40 °C urea begins to break down into cyanates that modify proteins at lysine (Stark et al., 1960). This explains why, at 35 and 40 °C, the data collected before 8 h show a decrease in exchange rates in 8 M urea, while at times >20 h (data not shown) the exchange rates in 8 M urea are greater than or equal to those in nondenaturing buffer.

Discussion

Enthalpy for Unfolding in 8 M Urea. The two-process model of NH exchange kinetics of single BPTI protons is corroborated by the enthalpy for unfolding of BPTI in 8 M urea. The value for unfolding in nondenaturing solvents cannot be obtained by conventional methods, as the unfolding temperature is too high. In 8 M urea, pH 4–8, the enthalpy of unfolding is 45 ± 5 kcal/mol, which agrees well with the value of ~45 kcal/mol from the hydrogen exchange data, obtained from an observed E^*_{app} for exchange of ~65 kcal/mol and an activation energy for the chemical exchange step of ~20 kcal/mol. The enthalpy of unfolding for (carboxamidomethyl)-BPTI of 47 kcal/mol (Vincent et al., 1971) is also in good agreement.

There Are Three pH-Dependent Forms of Folded BPTI. The most thermally stable form of folded BPTI exists over the pH range 4–8. At pH <2 and pH >10, there are different forms of the folded state of BPTI; both have lower thermal stability. The titration between the two acid pH forms of BPTI is shown in Figure 3. This figure gives the pH dependence of the negative ellipticity at 225 nm, which is proportional to the fraction of folded protein. The apparent pK for the transition is 3.2.

In 8 M urea, BPTI also undergoes a pH-dependent transition from a form that is uniformly stable from pH 4 to 8 to a less stable form at pH 2 (Figure 4). The apparent pK for this transition is also 3.2. However, in urea, the stability again increases on the acid side of pH 2 (Figure 4).

It is interesting that while the folded states of BPTI, at their respective pHs, have no detectable circular dichroic differences

the unfolded forms in urea are quite different; the unfolded state in urea at pH 4–8 apparently retains some secondary structure (Figure 2).

A similar low-pH transition has been reported for the thermal unfolding, measured by optical rotary dispersion, of the carboxamidomethylated BPTI. This derivative has the disulfide bond Cys-14–Cys-38 reduced, and a T_m of 73 °C at pH 5.5 (Vincent et al., 1971). These authors did not measure unfolding curves for the native inhibitor above pH 3. They observed for the carboxamidomethylated inhibitor a transition between pH 8 and 10 to a third form, less stable at the higher pH. Our data at pH 10 are consistent with this (Figures 1 and 2), but we did not examine this pH region in detail.

The acid pH dependence of thermal stability in KCl at constant temperature (Figure 3) is in very good agreement with the pH dependence of the stabilizing electrostatic free energies calculated by the method of Gurd and associates (Friend & Gurd, 1979) (J. Matthew and F. M. Richards, personal communication). The transition is likely due to the titration of Asp-50 and Glu-7 (J. Matthew and F. M. Richards, personal communication).

The pH dependence of the thermal stability of BPTI, together with the two-process model, now explains the unusual pH of the minimum rate, pH_{min}, as well as the shallowness of the pH_{min} region observed for the pH–rate profiles of single protons measured by NMR. For most native proteins, the pH_{min} for peptide NH's is around pH 3, and the rates become first order in OH^- or H^+ ion on either side of a sharp minimum. But for the slowest exchanging protons in BPTI, $pH_{min} \simeq 3.8$, and the minimum in the pH–rate curve is unusually flat. This at first led to difficulty in interpretation (Hilton & Woodward, 1978), but later became clarified along with the understanding that the exchange mechanism changes with pH and that, around pH_{min}, these protons exchange by a high E^*_{app} process (Hilton & Woodward, 1979) which we later assigned to major thermal unfolding (Woodward & Hilton, 1980).

The explanation for the pH dependence near the pH_{min} for the slowest exchanging protons is as follows. At pH ~4, the high E^*_{app} rates begin to be accelerated by increased unfolding of the protein with decreasing pH. This acceleration of the acid-catalyzed rates, relative to that of the base-catalyzed rates, increases the pH_{min}. But there is also an opposite effect, the slowing of the chemical exchange step, for which the pH_{min} is around 2.8. The net result for the pH–rate curve is a nearly pH-independent region between pH 3 and 4, and an apparent minimum at pH >3, as observed (Hilton & Woodward, 1979).[2]

Low E^*_{app} Exchange Rates Are Not Correlated to Thermal Stability in Urea. If rates of exchange from folded BPTI are correlated with thermal stability, then they should be accelerated in denaturants, such as 8 M urea, that accelerate the unfolding rate and decrease thermal stability of the protein. While the mechanism of thermal destabilization of proteins in urea is not certain, it is known that urea increases the unfolding rate in globular proteins by orders of magnitude and in proportion to the urea concentration (Warren & Gordon, 1970; Sharma & Hopkins, 1979). The thermal stability of BPTI is substantially decreased in 8 M urea (Figures 2 and 4).

Urea does not increase rates of exchange from the folded state of BPTI, but instead decreases them (Figure 7). The conditions for this experiment, pH 6.5, in the temperature range 20–35 °C were chosen to eliminate contributions from thermal unfolding.

In conclusion, there is no correlation between thermal lability in urea and exchange rates for the low E^*_{app} process, a dynamic process of the folded state. In the case of homologues and derivatives for which differences from the parent protein in thermal stability are introduced either by amino acid substitution or by chemical modification, there may be local changes in low E^*_{app} exchange rates due to alterations in packing, electrostatic interactions, etc., but we do not expect a global correlation between low E^*_{app} exchange rates and thermal lability.

In summary, the enthalpy of unfolding in 8 M urea, the pH dependence of thermal stability, and the absence of an acceleration of low E^*_{app} exchange rates after addition of 8 M urea support the two-process interpretation of the BPTI single proton exchange data. In that interpretation, the low E^*_{app} mechanism, but not the high E^*_{app} mechanism, involves motions of the folded state. Consequently, if one wishes to fit hydrogen exchange rates, activation parameters, pH dependence, etc. to a model of the dynamic structure of folded proteins, then one must refer to low E^*_{app} rates.

Mechanism of the Low E^*_{app} Process. The mechanism for the motions of folded proteins that limit hydrogen exchange is controversial (Woodward & Hilton, 1980; Englander et al., 1980; Wuthrich et al., 1980a). There are two limiting models. In one, solvent species penetrate the packed protein matrix by pathways provided by cavities that occasionally collect as a result of small movements of protein atoms, in the range of tenths to ~1 Å. Peptide–solvent proton exchange occurs inside the protein (Woodward & Rosenberg, 1971a; Woodward, 1977; Woodward & Hilton, 1980). A more specific suggestion of cavities created by mobile defects of constantly redistributing free volume arising from local oscillations in protein intramolecular bonding, including H bonding, has been made (Lumry & Rosenberg, 1975; Lumry, 1979). Richards (1979) has suggested that such mobile defects could arise from volume fluctuations of small cavities that arise from packing defects in proteins and that catalyst ions reach interior NH's through transient networks of hydrogen-bonded water in these cavities [discussion after Woodward & Hilton (1980)].

In the second type of model, larger scale motions expose buried NH's to bulk solvent where exchange takes place. Local unfolding of segments of secondary structure is proposed (Englander et al., 1980), as are translational and rotational motions of hydrophobic domains or clusters relative to each other (Wuthrich et al., 1980a).

Among the evidence we have cited in support of a model of penetration based on small motions, and against a local unfolding model, are the following. Over 90% of the peptide NH protons exchange in solution with low activation energy; >95% of peptide NH's exchange with deuterium in the crystal (Schoenborn et al., 1978); for lysozyme, exchange kinetics are identical in the crystal and in solution (Tuchsen & Ottesen, 1979; Tuchsen et al., 1980), and crystalline proteins are found to have small, but significant, atomic motions (Frauenfelder et al., 1979); denaturants do not accelerate exchange rates (Woodward et al., 1975a); interface protons of the trypsin–trypsin inhibitor complex exchange with the pH and tem-

[2] This explanation for the unusual shape of the pH–rate dependence of the slowest exchanging protons in BPTI is consistent with calculations of J. B. Matthew and F. M. Richards (personal communication). They have computed the pH-dependent electrostatic contributions to protein stability, as well as the possible effects of the local fields on the activation energy for exchange at individual amide positions. The estimated pH-dependent rate profiles fit quite well the observed exchange data for several individual protons in BPTI.

perature dependence characteristic of buried protons in globular proteins (Woodward, 1977); NH protons in small peptides, known not to be hydrogen bonded, have slowed exchange rates (Llinas et al., 1973; Brewster & Bovey, 1971); the conformational equilibria controlling exchange have low apparent enthalpy, 0–15 kcal/mol; O_2 diffuses rapidly through the protein matrix of globular proteins (Lakowicz & Weber, 1973); for the slowest exchanging protons in the BPTI β sheet, one pair of adjacent NH's has identical kinetic properties, one pair of adjacent NH's has quite different kinetics, and a third pair of oppositely oriented NH's has identical kinetics (Hilton & Woodward, 1979); molecular dynamics calculations suggest that small, picosecond, atomic fluctuations (a) are less probable for the β-sheet protons observed to have the slowest exchange rates (b) accommodate flips of interior aromatic side chains, and (c) provide penetration pathways for O_2 in myoglobin (McCammon & Karplus, 1980).

Arguments in support of a local unfolding model and against a penetration model are that as hydrogen exchange requires hydrogen bonding with solvent this necessitates the prior breaking of an internal H bond, and it is unlikely because of steric constraints that one H bond can break without the breaking of several adjacent hydrogen bonds, especially in sections of the secondary structure; that the observation of first-order difference exchange curves generated by partial labeling and curve substraction of ligand-sensitive exchange rates in hemoglobin indicates that each difference curve measures the rate of one local unfolding segment; and that the penetration of HO^- and H^+ catalyst ions into the protein interior is energetically very unfavorable (Englander et al., 1980).

Regarding the possibility of breaking only one H bond, although there is considerable uncertainty in gathering H-bond distances from X-ray crystal data for proteins, it seems very likely that there are, in folded proteins, a large number of internal hydrogen bonds sufficiently strained so that motions in the range 0.5–1.5 Å of the donor and/or acceptor atoms could break one H bond without necessarily breaking adjacent hydrogen bonds. We expect that such an occurrence may be facilitated by the breakage and formation of H bonds, perhaps bifurcated, by the donor or acceptor with mobile water molecules or with surrounding protein atoms, and/or by breakage and formation of H bonds in other regions of the protein. If some H bonds are broken only in pairs, they may still exchange inside the protein.

Gavish (unpublished experiments) has calculated the correlations between variations of the mean square displacement observed by Frauenfelder et al. (1979) and the density of stabilizing intramolecular interactions, particularly hydrogen bonds. He concludes that important contributions to protein dynamics should originate from the kinetics of formation and breaking of intramolecular hydrogen bonds and those resulting from protein–solvent interaction.

Regarding the significance of the difference hydrogen exchange data (Englander, 1975), we have suggested that the results may as well be explained as fractional labeling of many protons kinetically isolated from a broad distribution of rates and having apparent first-order kinetics only because a small enough time window is chosen [discussion after Englander et al. (1980)].

With regard to the energy barrier to the penetration of charged species into the packed interior, there are few applicable data. Richards has proposed transient networks of water molecules that serve as conduits for OH^- and H^+ ions from the solvent to buried NH's [Richards, 1979; discussion after Woodward & Hilton (1980)]. We suggest that, in addition, protein–solvent H bonding, as well as packing considerations, may be important in the creation of solvent penetration pathways. It is possible that either water networks or H^+ and OH^- ions hydrated with one to several water molecules move through the protein in pathways determined not only by the relaxation of surrounding atoms but also by the formation of transient H bonds between solvent atoms and protein atoms as the latter move in and out of the optimal H-bonding distance.

The hydrophobic cluster model of Wuthrich and Wagner rests on the observations that hydrogen exchange rates, but not aromatic ring flips, are correlated with thermal stability. As we posit that such a correlation with low E^*_{app} exchange rates has not been demonstrated, we expect that the motions that determine hydrogen exchange rates may be rather more like, than different from, those involved in interior aromatic ring flips.

Wuthrich et al. have proposed that the single proton exchange data for a thermally labile derivative of BPTI are incompatible with the two-process model [discussion after Woodward & Hilton (1980); Wuthrich et al., 1980b]. The BPTI derivative is reduced and aminoethylated at the Cys-14–Cys-38 disulfide bond, and T_m = 70 °C. Rates of the slowest exchanging protons for the derivative are 10^2–10^3 times faster than those for native BPTI over the pH range 0.5–10 at 22 °C. However, these data are not in contradiction with the two-process model, nor with the suggestion that the low E^*_{app} exchange rates are not correlated with thermal stability. For native BPTI, at temperatures around 50 °C, the relative values of the exchange rates by each process are such that, at high pH, the low E^*_{app} process dominates. The two-process model does not predict that this will also be true for a derivative which has $T_m > 25$ °C lower than that of the native BPTI. One expects, rather, that for the derivative the high E^*_{app} exchange rates are (a) much greater than the high E^*_{app} rates in native BPTI and (b) greater than the low E^*_{app} rates in the derivative even at high pHs and low temperatures. As the activation energies for the exchange rates at high pH in the derivative are not known, the data reported by Wuthrich et al. (1980b) do not rule out a model that the low E^*_{app} exchange rates are not correlated with thermal stability.

The absence of a correlation of thermal lability with rates for exchange from the folded state would imply that the comformational fluctuations of the folded state that limit low E^*_{app} exchange are not related to the motions involved in major thermal unfolding. As we envision it, for a single NH proton, the low E^*_{app} motions limit exhange rates only in collapsed, packed domains. As unfolding proceeds, and the lifetimes of the unfolded forms increase, the exchange mechanism for that NH switches as the rates for exchange from unfolded and/or intermediate species become more rapid than those for exchange from the folded substates. Proteins do undergo reversible motions in the process of unfolding, but we expect that these are not linked in frequency, amplitude, or cooperativity to the low E^*_{app} mechanism that allows most of the interior NH protons to interact with OH^-, H^+, and H_2O. If this is true, it argues against the hydrogen exchange models of local unfolding and of hydrophobic clusters for the low E^*_{app} mechanism. Local unfolding, motions of hydrophobic clusters, or both may operate in the high E^*_{app} process.

The contribution of protein dynamics to protein thermodynamics is a question related to the mechanism of the low E^*_{app} exchange process. In a model of solvent penetration based on small fluctuations, the effect of mobile water mol-

ecules in a packed protein interior of fluctuating atoms could be to maximize hydrogen bonding of buried atoms by flexible interactions that do not require rigid maintenance of protein atom donor–acceptor geometry. Water molecules moving through the protein interior could take up the free-energy slack occasioned by fluctuations of H-bond donor and acceptor atoms, and thereby stabilize the dynamic folded conformation. This view is consistent with the observation of Vinogradov (1979) in his review of the internal water in proteins suggested by the X-ray crystal data. Vinogradov points out that internal water molecules occur singly as well as in clusters of two to seven, that they form extensive H-bond interactions with buried protein atoms, that the partial occupancy of most apparent water sites suggests a dynamic nature of the water structure, and that internal water offers structural flexibility and thermodynamic stability. The suggestion of transient polar interactions between protein atoms and penetrating water species is compatible with the models of mobile defects (Lumry & Rosenberg, 1975) and internal cavity volume fluctuations (Richards, 1979).

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